In Vitro Evaluation of the Activity of 2'-ß-L-Deoxythymidine (LdT; telbivudine) and 2'-ß-L-Deoxycytidine (LdC) against Lamivudine-Resistant Mutants of Hepatitis B Virus

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1. SUMMARY

The objective of this study was to determine the *in vitro* activity of the investigational drugs LdT (telbivudine) and LdC against lamivudine-resistant mutants of Hepatitis B virus (HBV). The biologically relevant mutants of HBV (subtype ayw) that have been determined to confer resistance to lamivudine therapy in the clinical setting arise in the polymerase gene and comprise two single mutations, M552V and M552I, found in the key YMDD active site motif as well as two double mutants L515M/M552V and L515M/M552I. [Note that the L515M mutant referred to in this study is equivalent to the L526M or L528M mutations in the B domain often cited in HBV resistance studies: the difference in numbering reflects sequence insertions/deletions among different HBV genotypes].

Recombinant DNA constructs corresponding to these lamivudine-resistant mutants were created by site directed mutagenesis. The test system comprised stable cell lines harboring each of the transfected mutant genomes, as well as a wild-type control HBV genome. The antiviral activity of LdT (telbivudine) and LdC, together with the control drugs lamivudine and PMEA, was determined against the mutant and wild-type HBV genomes in the respective test cell lines.

LdT (telbivudine) and LdC demonstrated minimal activity against the M552I single mutant or the L515M/M552V and L515M/M552I double mutants, although LdC did reach an EC_{50} against the L515M/M552V mutant. However, in contrast to lamivudine, both drugs retain almost full antiviral activity against the M552V single mutant HBV genome. The M552V mutation is significantly resistant to lamivudine and is thought to be a key intermediate in the major pathway for development of breakthrough resistance to lamivudine. In lamivudine-treated patients, the M552V mutant typically appears 4 to 8 weeks prior to the emergence of the highly resistant L515M/M552V double mutant (Gauthier et al, 1999), which reportedly accounts for 60-70% of all lamivudine resistance in hepatitis B patients (Ahmed et al, 2000).

These laboratory results suggest that the activity of LdT (telbivudine) and LdC against the M552V mutant may help suppress a major proportion of YMDD-mediated emergence of antiviral resistance in patients. Coupled with the better quantitative suppression of HBV replication observed for LdT treated patients (compared to lamivudine recipients) in the current Phase IIB clinical trial, these results suggest that YMDD-mediated HBV resistance to LdT is likely to be substantially less frequent than is observed with lamivudine treatment.

2. INTRODUCTION

In controlled clinical studies of lamivudine (100 mg qd), administered to HBV-infected patients, the prevalence of YMDD-mutant HBV was 14 to 32% after one year of treatment and as much as 58% after two to three years of treatment. Mutant virus was associated with evidence of diminished treatment response relative to lamivudine-treated patients without YMDD mutations.

Genotypic analysis of viral isolates obtained from patients with renewed HBV replication while receiving lamivudine suggests that a reduction in HBV sensitivity to lamivudine is associated with mutations resulting in a methionine to valine or isoleucine substitution in the YMDD motif of the catalytic domain of HBV polymerase (position 552) and a leucine to methionine substitution at position 515 or 528 (depending on the genotype/subtype of HBV).

At the present time, there is no cell-based HBV infection system that can be used to assess the activity of antiviral agents against cells infected with lamivudine-resistant HBV isolates from patients. The DHBV *in vitro* model has not proved useful to select drug-resistant mutations because the primary duck hepatocytes used in this model cannot be sustained for more than a few weeks in cell culture. The relevance of selection of drug-resistant mutants in the woodchuck *in vivo* model is dubious because the spectrum of lamivudine-resistant mutants in the woodchuck does not match that identified in HBV-infected patients.

HBV recombinants containing the YMDD mutation can be used to generate cell lines expressing lamivudine-resistant HBV, although these genomes are often less replication-competent than wild-type HBV *in vitro* (Fu and Cheng, 1998). These recombinant systems provide the best approach to addressing the question of resistance. We initially tried to characterize the activity of our investigational drug LdT (IND 60,459) against lamivudine-resistant HBV strains (YMDD mutants) through the use of transient transfection assays using Huh-7 liver cells, the preferred cellular system for such assessments, according to the approach described by Ono *et al*, 2001. In Huh-7 cells, we were surprised to see poor activity of LdT against even wild-type HBV. Further investigation revealed that, while Huh-7 cells phosphorylate lamivudine quite well, there was minimal phosphorylation of LdT to the active triphosphate. These results were summarized in the materials submitted to the Agency for the LdT End of Phase 2 Meeting (IND 60,459; Serial 024). Based on this experience, we have concluded that the system of Ono *et al* is not appropriate for the analysis of our drugs.

As an alternative approach we introduced mutated HBV genomes into HepG2 cells via transient transfection. These experiments were similarly unsuccessful, primarily due to the poor and variable transfectability of these cells, which precluded obtaining consistent values for antiviral efficacy.

We thus concluded that a more appropriate way to address the resistance question for LdT and LdC is by using stable HepG2 cell lines expressing recombinant HBV viruses with wild-type genomes as well as lamivudine-resistance mutations at positions 552 and 515 or 528, respectively. Compared to the preceding methods, these stable cell lines offer consistent polymerase expression levels combined with a good signal-to-noise. In the present report, we describe these experiments and the results obtained.

3. PROCEDURES

3.1 Transfections of HepG2 Cells to Create Lamividine-Resistant Stable Cell Lines.

A. Objective

To create stably transformed cells harboring the chararacteristic lamivudine-resistant and wild type HBV genomes and to test the activity of LdT and LdC against the mutants along with lamivudine and adefovir as controls.

B. Reagents

Media: HepG2 Growth Media

EMEM (Mediatech, Cat#MT 10-010-CV)
10% FBS (Mediatech, Cat#MT 35-011-CV)
1x L-glutamine (2mM final)
1x Penicillin-Streptomycin (100 I.U./ 100 μg per ml final)
1x Na-Pyruvate (1mM final)
1x Non-Essential Amino Acids (NEAA, 0.1mM final)

HepG2 Transfection Media

EMEM (Mediatech, Cat#MT 10-010-CV)
10% FBS (Mediatech, Cat#MT 35-011-CV)
1x L-glutamine (2mM final)
1x Na-Pyruvate (1mM final)
1x NEAA (0.1mM final)

HepG2-Stable Cell Line Growth/Selection Media

EMEM (Mediatech, Cat#MT 10-010-CV)
10% FBS (Mediatech, Cat#MT 35-011-CV)
1x L-glutamine (2mM final)
1x Penicillin-Streptomycin (100 I.U./μg per ml final)
1x NaPyruvate (1mM final)
1x NEAA (0.1mM final)
500 μg/ml Geneticin (G-418, Life Technologies Cat# 10131)

Constructs: pCMV-WT (ayw)
pCMV-M552V
pCMV-M552I
pCMV-L515M/M552V
pCMV-L515M/M552I
pCMV-neo

All constructs contain the HBV genome cloned behind the CMV promoter. HBV plasmids containing point-mutated polymerase genes were derived by site-directed mutagenesis using pCMV-hbv as parent and a commercial kit (Stratagene's QuikChange kit, Cat.# 200518-5) as described previously (Allen et al., 1998). pCMVhbv (kindly provided by Dr. C. Seeger, Fox Chase Cancer Institute) contains an overlength HBV genome subtype ayw. The construct containing a single L515M mutation was not generated, because this mutation is thought to confer only minimal resistance to lamivudine; rather it serves as a compensatory mutation (Gauthier et al, 1999). Plasmid pCMV-neo was used to confer resistance to G-418 antibiotic. (neomycin) This plasmid contains the backbone of pEGFP-N1 (Clontech Cat# 6085-1) with the SV40-driven Kan^r/Neo^r expression cassette but without the EGFP expression cassette.

1. *Preparation of cells for transfection*: Cells were plated in collagen-coated 6-well plates (Biocoat, Becton Dickinson, Cat-# 35-4400) at $2x10^5$ cells per well in 3ml HepG2 growth media. Cells were incubated overnight at 37°C.
2. *Preparation of Fugene:DNA complex*: Each construct was used to transfect 2 wells of a 6-well collagen-coated plate. Controls included 1 well containing the pCMVhbv wild-type construct without pCMV-neo DNA and 1 well containing the neo^R -plasmid only. Cells were transfected with Fugene (Roche cat#1815091) according to the manufacturer's suggested protocol with a Fugene-to-DNA ratio of 3:1 (3ul Fugene +1ug of supercoiled plasmid DNA/well). Briefly, 6ul of Fugene was diluted into 200ul of serum free EMEM medium in a microcentrifuge tube. 2ug of the respective HBV plasmid DNA along with 0.2ug of pCMVneo DNAwas then added. . The solution was gently mixed and then incubated for 15 minutes at room temperature.
3. *Transfection of cells*: 6-well plates were aspirated and fed with 2ml HepG2 transfection media. The Fugene:DNA complex solution was then added slowly to the cells while swirling the plate to evenly disperse the solution. The plates were incubated overnight at 37°C. HepG2-Stable Cell Line Growth/Selection Media was added the following day.

3.2 Selection of Stable Cell Line Colonies and Subclones

1. Transfected cells were fed twice a week for 2 ½ weeks until distinct G418-resistant colonies formed.
2. Colonies that appeared to be "clonal" (not in contact with any other colonies) were picked off the 6-well plate with a pipette tip and placed in a BD 96- well

collagen plates in 150 µl of HepG2-Stable Cell Line Growth/Selection Media. 16 colonies were picked per construct.

3. Media was changed every 3-4 days. HBV expressing colonies were identified via testing of 100 µl of culture supernatant for presence of HBeAg via ELISA.(see below)
4. Positive colonies were subcloned by limiting dilution in collagen 96-well plates and culture supernatant was screened by ELISA 2 weeks later (media was changed every 3-4 days).
5. Positive wells were expanded, frozen stocks were made and cell line was then subcloned again by limiting dilution and the culture supernatant was screened by ELISA 2 weeks later.

3.3 Testing of Stable Transfected Cell Lines

Stable cell line clones were screened for expression of the viral genome by two tests. The first assay was a semi-quantitative ELISA assay that measures the production of hepatitis B virus e antigen (HBeAg), a viral protein marker that correlates well with viral replication. Cell lines that produced high levels of HBeAg were then tested for the production of replicative viral genomes using the endogenous polymerase assay.

1. *HBeAg-ELISA of Stable Cell Line Culture Supernatant*: The capture antibody was a mouse anti-HBeAg mAb used at 10ug/ml. 100ul of culture supernatant was used directly from 96-well cell culture plate. Detection antibody was a polyclonal (rabbit) anti-HBc/eAg- antibody (DAKO, Cat# B0586) at 1:3,000 dilution in 10% FCS/TNE). Peroxidase-conjugated goat anti-rabbit-IgG (1:10,000; Zymed Cat# 81-6120)) was used to develop and substrate was o-phenylenediamine (Zymed Cat# 00-2003) in citrate/phosphate buffer. Development was stopped with 2N Na_2SO_4 prior to reading the optical density (O.D.) at 490nm in a Fusion plate reader (Packard Instuments).

2. *Endogenous Polymerase Assay (EPA) of cell lysate*: Cells were grown in 12-well collagen plates to 100% confluency for 3-4 days and cytosolic lysates were prepared in 1ml of 50mM Tris-HCl pH 7.4, 150mM NaCl, 5mM $MgCl_2$, 0.2%NP-40. EPAs were essentially performed as described (Seifer et al., 1998). Briefly, intracellular HBV nucleocapsids were immunoprecipitated from the cytoplasmic lysates overnight at 4°C with a polyclonal rabbit anti-HBc/eAg antibody (DaKo Cat# B0586) and immobilized on protein A sepharose CL-4B beads (Amersham Pharmacia Cat# 17-0780-01). The immobilized capsids were then washed, endogenous polymerase reactions were initiated in 50ul reaction volume containing 50mM Tris-HCl pH7.4, 75mM NH_4Cl, 1mM EDTA, 20mM $MgCl_2$, 0.1mM β-ME, 0.5%NP-40, 100 µM cold dGTP, TTP, dCTP, and 50 nM ^{33}P-dATP (Perkin Elmer Life Sciences Cat# NEG 612H), and incubated overnight at 37°C. Following digestion with 1mg/ml of Proteinase K (Roche, Cat# 1373196) for 1h at 37°C, ^{33}P-labeled HBV DNA was liberated via phenol/chloroform extraction The nucleic acids were finally precipitated with equal volumes 5M NH_4-acetate and 2.5 volumes 100% EtOH and separated on a

1% native agarose gel in Tris-borate buffer. Gels were either fixed with 7%TCA, then dried, or blotted onto positively charged nylon membrane (Pall Biodyne Plus Cat# 60406) overnight at room temperature via capillary transfer in 0.4 N NaOH.. Dried gels/membranes were exposed to a phosphoimager screen (Molecular Dynamics) overnight at room temperature, then scanned (Storm 860, Molecular Dynamics) and quantitated with ImageQuant (Molecular Dynamics) software.

The clonal cell lines that were selected as having high HBeAg expression as well as high level production of replicative genomes were designated as follows:

Cell line	Virus
WT3/C1	Wild-type (ayw)
V1/C9	M552V
I2	M552I
MV5/B3	L515M/M552V
MI4	L515M/M552I

3.4 Antiviral Testing

3.4.1 Test Articles

LdT	Lot# 1.004E (Idenix)
LdC-HCl	Lot# LP017/02PG (Idenix)
Lamivudine	Lot# LP017/02PG (Moravec)
PMEA	Lot# 208135-000 (Moravec)

PMEA is the active component of the prodrug adefovir

3.4.2 Antiviral Testing Procedure

1. 12-well Biocoat collagen I plates (Becton Dickinson Cat#35-4500) are seeded with cells at a density of 0.5-1x10^6 cells per well in 2ml DMEM containing 5% FBS, 2 mM L-glutamine, 100 I.U. penicillin/100 µg/ml streptomycin, and 0.5mg/ml G-418.

2. Drug dilutions are prepared freshly in 100% DMSO as 200x stocks. For each experiment, 4 aliquots of each drug dilution series are stored at –20°C until used. . Once cells reach confluency, drug treatment is initiated by adding 10 µl of drug dilution into 2ml of fresh DMEM + 5% FBS. Thus, the final DMSO concentration does not exceed 0.5% . The no-drug control well receives only 10 µl of DMSO. Cells are treated every other day with 2 ml of fresh drug/medium for a total of 8 days. Cell lysates are then collected on day 10 as described below.

3. Aspirate media and rinse cell monolayer carefully once with 1 ml of PBS.

4. Add 1 ml of lysis buffer (50 mM Tris-HCl pH 7.5/150 mM NaCl/5 mM $MgCl_2$/0.2% NP-40). Store on ice for 30 min to 4 h.

5. Harvest lysed cells. Transfer to 1.5 ml microfuge tubes.

6. Clarify lysate by spinning for 5 min at RT and 14,000 rpm.

7. Transfer clarified lysate to fresh tubes. Snap-freeze on dry-ice and store at -80°C until ready to continue with endogenous polymerase assays essentially as described above.

8. EC_{50}s were generated from the phosphorimager data by curve fitting using Xlfit software.

4. RESULTS

Table 1 summarizes the results obtained when LdT and LdC, as well as the lamivudine and PMEA controls, were tested for activity against the different mutant and wild-type HBV genomes expressed in stably transfected HepG2 cells. The antiviral activities are presented in Table 1A. The EC_{50} values obtained for the different drugs against the wild-type virus generally accord well with literature-reported values, except that the mean EC_{50} value for LdT is higher than the typical value of around 200 nM seen in most of our prior studies. We have previously noted that the antiviral activity of LdT in cell culture is quite variable and that the potency of LdT in cell culture assays does not seem to be predictive of the efficacy seen in patients in the clinical setting.

With respect to the activity observed against lamivudine-resistant mutants, we found that PMEA retained significant activity against all the mutants in agreement with prior reports (see Gilead FDA briefing document, 2002). Against the two double mutants or the M552I single mutant, LdT, LdC and lamivudine were largely inactive ($EC_{50} > 1$ mM), although LdC showed marginal activity against the L515M/M552V mutant with an EC_{50} of approximately 780 μM. The major finding from the present study was that LdT and LdC retained almost complete activity against the M552V single mutant, whereas the activity of lamivudine against this mutant was significantly diminished.

The effect of the lamivudine-resistant mutants on the observed efficacy of the drugs can best be seen from the fold-resistance analysis presented in Table 1B. The results obtained in this study are in broad agreement with prior studies (as summarized in the Gilead FDA Advisory Committee Briefing Document, 2002). It is clear from Table 1B that LdT, LdC and lamivudine show a substantial fold resistance when tested against either of the double mutants or the M552I single mutant. However, the status of the single M552V mutant is very different. LdT and LdC exhibit essentially unchanged antiviral activity against this mutant, with respective fold resistance changes of 1.2 and 2.1 fold, whereas lamivudine shows a 24.8 fold resistance in our hands and a 153 fold resistance according to the Glaxo group (Allen et al, 1998)

5. CONCLUSIONS

The data described above provide a clear picture of the overall *in vitro* resistance profile for Idenix's two investigational drugs LdT (telbivudine) and LdC.

Both LdT and LdC were relatively inactive against the double-mutant HBV strains commonly found in hepatitis B patients with established lamivudine resistance. If these *in vitro* results are predictive of clinical activity, then the results suggest that LdT and the LdC prodrug currently being investigated in the clinic may have minimal anti-HBV activity in patients with established lamivudine resistance, harboring the double-mutant HBV strains. However, two recent abstract reports in the literature have highlighted the problem that results with laboratory transfectants sometimes have poor predictive value with regard to activity in the clinic. A report from Gilead virologists (Delaney *et al.*, 2001) suggested minimal *in vitro* activity for entecavir against YMDD-mutant HBV strains, while another abstract at the same meeting (AASLD 2001) described the results of a large prospective trial conducted by entecavir's sponsor (Bristol Myers Squibb), in which entecavir treatment produced substantial HBV DNA reductions in lamivudine-resistant hepatitis B patients (REF xxx). Thus, in view of the problematic clinical predictive value for HBV-related laboratory results, it may be desirable to perform a small clinical trial for LdT (and LdC) in patients with lamivudine-resistant HBV, despite the minimal *in vitro* activity for these two compounds against double-mutant HBV strains.

Most interestingly, the laboratory studies reported here demonstrated essentially unaltered anti-viral activity for LdT and LdC against the M552V HBV mutant, in contrast to lamivudine. The M552V mutation is critical for the development of lamivudine resistance, as it is thought to be the first step in the pathway that leads to the M515L/M552V double mutant, which accounts for 60-70% of lamivudine resistance in hepatitis B patients (Ahmed et al, 2000). Idenix therefore believes this *in-vitro* finding is important to the overall understanding of the potential resistance profile of LdT (telbivudine), as activity against the key M552V mutation may help to suppress the emergence of viral resistance in hepatitis B patients being treated with LdT (telbivudine).

While clinical antiviral resistance patterns can be established only from clinical trials, the unaltered activity of LdT and LdC against M552V HBV mutants, coupled with the better quantitative suppression of HBV replication observed for LdT treated patients in the current Phase IIB clinical trial, suggest that YMDD-mediated HBV resistance to LdT is likely to be substantially less frequent than is observed with lamivudine treatment.

The pattern of clinical antiviral resistance is, of course, best determined by virologic studies using samples from clinical trials, particularly from patients exhibiting virologic breakthrough on-treatment. Idenix' Phase II and III clinical protocols for LdT and for an LdC prodrug (val-LdC) are incorporating this approach, i.e. characterization of the *de novo* resistance patterns to LdT and LdC through molecular genotyping of HBV DNA amplified from patients exhibiting virologic breakthrough during study treatment.

Table 1. Inhibition Profile of LdT (telbivudine), LdC, Lamivudine and PMEA against wild-type and lamivudine-resistant mutant HBV viruses derived from stable cell lines as determined by EPA of intracellular nucleocapsids. A. Antiviral efficacy; B. Fold resistance

A. Antiviral Efficacy

Cell Line	Virus	Drug			
		LdT	*LdC-HCl*	*Lam*	*PMEA*
		EC_{50} (uM)	*EC_{50} (uM)*	*EC_{50} (uM)*	*EC_{50} (uM)*
WT3/C1	WT (ayw)	0.65 ± 0.28	0.18 ± 0.09	0.05 ± 0.03	0.33 ± 0.17
V1/C9	M552V	0.85 ± 0.48	0.36 ± 0.13	0.96 ± 0.36	1.02 ± 0.22
I2	M552I	≥1000	≥1000	≥1000	1.6 ± 1.12
MV5/B3	L515M/M552V	≥1000	777 ± 99	≥1000	0.62 ± 0.3
MI4	L515M/M552I	≥823 ± 307	≥1000	≥1000	1.49 ± 0.3

B. Fold Resistance

Cell Line	Virus	Drug			
		LdT *X Resist.*	*LdC-HCl* *X Resist.*	*Lam* *X Resist.*	*PMEA* *X Resist.*
WT3/C1	WT (ayw)	1	1	1	1
V1/C9	M552V	1.2 ± 0.4	2.1 ± 0.5	24.8 ± 17.8	3.8 ± 2.3
I2	M552I	≥1360 ± 363	≥6733 ± 4245	≥22922 ± 9063	4.6 ± 3.0
MV5/B3	L515M/M552V	≥1360 ± 363	5051 ± 2717	≥22922 ± 9063	3.3 ± 2.9
MI4	L515M/M552I	≥1049 ± 226	≥6733 ± 4245	≥22922 ± 9063	3.6 ± 1.1

All numbers in both tables represent average values +/- SD derived from three to four independent experiments.

EC_{50} = effective concentration that reduces virus production by 50% in cell culture

Fold resistance = EC_{50} for the mutant HBV divided by the EC_{50} for the wild-type HBV

6. REFERENCES

Ahmed SNS, Tavan D, Pichoud C, et al. Early detection of viral resistance by determination of hepatitis B virus polymerase mutations in patients treated by lamivudine for chronic hepatitis B. Hepatology 32: 1078-1088, 2000

Allen MI, Deslauriers M, Webster A, et al. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Hepatology 27 (No. 6): p. 1670-1677, 1998.

Delaney WE, Huiling Y, Westland CE, et al. In vitro cross resistance testing of adefovir, entecavir, and β-L-thymidine (L-DT) against drug-resistant strains of HBV. Hepatology 34 (No.4, Pt 2): p. 628A, abstract #1825, 2001

Fu L and Cheng, YC. Role of additional mutations outside the YMDD motif of hepatitis B virus polymerase in L(-)SddC (3TC) resistance. Biochem Pharmacol. 55: 1567-1572, 1998.

Gauthier J, Bourne EJ, Lutz, MW et al. Quantitation of hepatitis B viremia and emergence of YMDD variants in patients with chronic hepatitis B treated with lamivudine. J. Infectious Dis 180: 1757-62, 1999.

Gilead FDA Advisory Committee Briefing Document. Adefovir dipivoxil for the treatment of chronic hepatis B. NDA 21-449. Table 1 p12. 5 July, 2002

Ono SK, Kato N, Shiratori Y, *et al*, The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J. Clin Invest. **107:**449-455, 2001

Seifer M, Hamatake R, Bifano M, Standring DN. Generation of replication-competent hepatitis B virus nucleocapsids in insect cells. J. Virol. 72: 2765-2776, 1998.